Water parameters and hydrodynamics in rivers and caves hosting *Astyanax mexicanus* populations reveal macro-, meso- and micro-habitat characteristics

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Abstract (<350 words)

The Mexican tetra (*Astyanax mexicanus*) has emerged as a leading model for evolutionary biology and the study of adaptation to extreme subterranean environments. The river-dwelling morphs of the species are distributed in Mexico and Texas, while the blind and cave-adapted morphs inhabit the karstic caves of the Sierra Madre Oriental in northeastern Mexico. Although the molecular, cellular and genetic underpinnings of *Astyanax* cavefish evolution are being elucidated, our understanding of its habitat and environment remains limited, thereby constraining the interpretations of its morphological, physiological, and behavioral adaptations. In particular, the physico-chemical parameters of the water and the hydrological regimes to which cavefish are subjected remain largely unexplored. From 2009 to 2024, we have recorded the physico-chemical parameters of the water in which *Astyanax mexicanus* cavefish and surface fish are found in the El Abra and La Colmena regions of the states of San Luis Potosí and Tamaulipas, Mexico. Data were collected using a variety of devices and probes, including both point and longitudinal measurements of water quality. A total of 13 caves out of the 33 known *Astyanax* caves and 27 surface stations (rivers, springs, ponds) were sampled. The comparison of epigean and hypogean waters showed strong signatures of the two macro-habitats. As compared to surface, cave water was fresher, much less conductive and highly anoxic. Moreover, at the meso-habitat level, a comparison between caves revealed significant differences in both specific water parameters and hydrological regimes. One- or two-year longitudinal recordings demonstrated that some caves exhibited relatively stable hydrological regimes, while others experienced multiple, sudden and significant fluctuations. Finally, distinct pools within a single cave showed notable differences, displaying a highly reproducible increase in water temperature as a function of depth or distance from the cave entrance, and revealing specificities at the micro-habitat level. Our comprehensive dataset on cave water quality and hydrodynamics is interpreted in the context of an integrated view of cave biology and the evolution of cave organisms. Our findings have significant implications for understanding the evolution of cavefishes and the biology of the species in general.

Introduction

The environmental parameters of an ecosystem constitute a fundamental source of information for the delineation of a species' ecological niche. The range of these parameters crossed by the range of the species is used to determine the preferred scales of each parameter for that species. In aquatic environments, water is the surrounding medium in which all organisms live, so the physico-chemical characteristics of the water are the most important to be studied in order to improve the knowledge of the range of parameters of the species, hence its biology. An understanding of these parameter ranges is essential for elucidating the species' adaptive responses to its environment, evolutionary history, and distribution within the ecological niche. They are of paramount importance for conservation issues and serve as the foundation for effective husbandry, health, and welfare of the species in captivity. This is particularly relevant when the species is utilized as an animal model in laboratory settings, where reproducible experiments are required across a diverse range of disciplines, including biomedical research.

The fish *Astyanax mexicanus*, or Mexican tetra (Sardinita Mexicana and Sardinita ciega), has become an excellent model for Eco-Evo-Devo, a concept first coined by Gilbert in 2015 (Gilbert et al., 2015). This is because this single species exists in two forms, or eco-morphs: freshwater river-dwelling morphs that are widely distributed in Mexico and the southern United States; and blind, depigmented dark-adapted morphs that inhabit the 33 caves discovered so far in a restricted area of northeastern Mexico (Elliott, 2018; Espinasa et al., 2018; Espinasa et al., 2020; Jeffery, 2001; Jeffery, 2008; Jeffery, 2009; Miranda-Gamboa et al., 2023; Mitchell et al., 1977). Of the more than 300 currently known species of cave and groundwater fishes (Proudlove, website), *Astyanax mexicanus* is by far the most studied (Rétaux and Jeffery, 2023) because the availability of the two lineages, surface and cave, offers unique opportunities for evolutionary biology and genetics (Casane and Rétaux, 2016; Gross et al., 2023; Gross et al., 2009; Protas et al., 2008; Protas et al., 2006). In the last twenty years much has been discovered about the neurosensory, behavioral and physiological adaptations of *Astyanax* cavefish, e.g. (Aspiras

et al., 2015; Blin et al., 2024; Hinaux et al., 2016; Riddle et al., 2018; Yoshizawa et al., 2010; Yoshizawa et al., 2014) and progress has been made in understanding the evolution of its genome (Krishnan et al., 2022; Leclercq et al., 2024; McGaugh et al., 2014; Warren et al., 2021). However, population biology studies have sometimes yielded conflicting results on the origin, structure, and age of cavefish populations (Bradic et al., 2012; Fumey et al., 2018; Herman et al., 2018; Policarpo et al., 2021; Policarpo et al., 2024; Strecker et al., 2004) and laboratory and field studies are sometimes difficult to reconcile (e.g. (Blin et al., 2024) vs. (Blin et al., 2020); (Aspiras et al., 2015) vs. (Espinasa et al., 2017; Simon et al., 2017)).

The need for an integrated view of the evolution of troglomorphy in cavefish during adaptation to the major environmental shift that life in caves represents has been raised (Rétaux and Jeffery, 2023; Torres-Paz et al., 2018). This includes a better understanding of the ecosystems in which *Astyanax* live, starting with long-term monitoring and comparison of surface and cave water quality. To our knowledge, very few studies have longitudinally documented water quality in caves in relation to cavefish biology, except to discuss the variation of condition factor across seasons for *Ituglanis* sp. in Brazilian caves (Bichuette and Trajano, 2021), or to discuss the evolution of temperature preference in some *Astyanax* cavefish populations (Tabin et al., 2018). The hydrological regimes are also largely unknown. The 2004 report by Fish (corresponding to his PhD thesis in 1976) describes the karst hydrogeology and geomorphology of the Sierra de El Abra region, which includes some of the *Astyanax* caves, and provides some temperature and pH data from surface and cave stations, but the work is disconnected from the biological perspective of the *Astyanax* cavefish (Fish, 2004). To fill this gap, here we present the results of both point and longitudinal measurements of water physicochemical parameters in several cave and surface localities where *Astyanax mexicanus* fishes swim. We report large, sometimes unexpected differences in water quality and hydrodynamics between surface

streams and caves, between caves, and between pools within a cave. We then discuss our results in terms of the evolution and biology of *Astyanax* Mexican cavefish.

Results

Between 2009 and 2024, we have systematically recorded the physico-chemical parameters of the water in which *Astyanax mexicanus* cavefish and surface fish swim. We collected data using several devices and probes (see Methods), with both point and longitudinal measurements of water quality. We sampled 13 caves out of the 33 known *Astyanax* caves and 27 surface stations (rivers, springs, ponds), mainly in the El Abra and La Colmena regions of the states of San Luis Potosi and Tamaulipas, Mexico (**Fig.1AB**, **Fig.S1** and **Supplemental File1**).

Comparing physico-chemical parameters of the water at the surface and in underground caves

Over the years, we have carried out 183 point measurements in caves and 77 point measurements in surface waters for temperature, pH and electrical conductivity (EC, abbreviated "conductivity" in the text below)(**Fig.2A** and **Table 1**, first two lines). These point measurement were taken mainly in February/March. The temperature in surface waters was on average 1.24°C higher than in caves (mean surface 24.86°C; mean cave 23.59°C; t = -3.8, df = 101.8, p-value = 1.8e-5), ranging from 16.8°C (Sótano del Arroyo, 2024) to 27.5°C (Cueva Chica, 2024) in caves and from 19.3°C to 31.1°C at the surface. The pH was very similar in the two environments (mean 7.67 in caves *vs* 7.78 in surface waters; t = -1.3, df = 98.3, p-value = 0.18). The conductivity was twice higher in surface water (mean 1092 µS.cm) than in cave water (mean 508 µS.cm; t = -10.7, df = 68.1, p-value = 2.5e-20). Therefore, epigean and hypogean waters are globally different and have a strong physico-chemical signature, especially in terms of conductivity and, to a lesser extent, temperature.

Despite significant differences emerging between the two conditions, we noticed a large dispersion of the data in our sampling (**Fig.2A**). For example, conductivity in surface waters varied from 144 µS.cm (in station #4 on map in **Fig.1A;** small pond near Camino Taninul, 2013) to 2034 µS.cm (station #15; Arroyo Los Otates, spring/resurgence close to la Cueva del Fraile, 2019), i.e., a 14-fold variation. Similarly, the same small pond near Taninul (station #4) displayed an exceptionally high pH value of 9.7 – but note that despite this extreme condition, a number of surface *Astyanax* swam there together with many big black tadpole of an undetermined anuran species. We reasoned that such data dispersion might be due to different hydrologic conditions encountered at different surface locations, and we replotted the data after sorting the sampling sites into “running” and “still” water. The term "running" is used here to include any river station with flowing water as a typical lotic ecosystem. The term "still" is used to include any other water unit such as a pond (including a pond in a river during the dry season), a lake, or a well, as a typical lentic ecosystem (**Fig.2B** and **Table 1**, last two lines). The mean temperature was globally similar in still and running water at the surface (24.92°C *vs* 24.66°C), and higher than in caves (23.59°C). The mean pH was higher in still water than in running water (8.24 *vs* 7.66; p=0.00019). Also, on average caves and flowing surface rivers display the same mean pH (7.64 and 7.65, respectively). Conversely, regarding conductivity still water bodies were similar to caves (545 µS.cm and 508 µS.cm) and both were very different from running water (1256 µS.cm; p=5.2e-8). In summary, the caves, the ponds and the rivers of the sampled area have different combinations of physico-chemical water parameters, and some extreme values can be observed. All the waterbodies we sampled hosted *Astyanax mexicanus* fish (surface- or cave-dwelling morphs), suggesting that the species can adapt to rather diverse ecological conditions (see also below).

Stability of measurements over time (1953-2024)

Finally, we sought to test whether the parameters analyzed might have varied over time. To this end, we compared our data (2009-2024) to earlier measures reported in bibliography (Bonet, 1953; Elliott,

2018; Fish, 2004; Mitchell et al., 1977; Ornelas-Garcia et al., 2018; Tabin et al., 2018; Johnson, 1967). One hundred and fifteen one-off measures were found between 1953 and 2022, in 8 different papers, mostly regarding temperature. A majority of these measures were found in Fish 1977 and 2004 (n=65), Tabin 2018 (n=17), Elliott 2018 (n=14) and Johnson 1967 (n=8). For our comparison, we chose one cave (Pachón) and one surface site (Nacimiento del Río Choy; station #3 on map **Fig.1A**), i.e. the stations where the most repeated samples are available over the years and where the exact sampling location is unambiguous. For conductivity, there is no data in the literature. For pH, measures done by Fish in 1977 are similar to ours - but note data from Fish are mostly from June and July (beginning of rainy season) whereas our data are from February-March (dry season). Regarding temperature, the old measurements showed values in the same range as our recent ones, both for Pachón Cave and for the Nacimiento del Río Choy spring (**Fig. 3**, see legend), suggesting that temperatures in these two sites have remained stable over 7 decades. The sparser data available from 4 other caves show the same trend (**Fig. S1**, see also legend).

Comparing physico-chemical parameters of the water between different caves, and between different pools in a single cave

Recent field studies in different caves have shown that the biology and behavior of cavefish can vary significantly (Blin et al., 2020; Espinasa et al., 2021; Hyacinthe et al., 2023; Simon et al., 2017), suggesting that cavefish may adapt to local environmental conditions. Since water is one of the most important environmental variables, we investigated whether temperature, pH and conductivity can vary significantly between caves. Here, we analyzed data recorded in several pools of 6 different caves (**Fig.1A** and **Supplemental File1**): Cueva de El Pachón, Cueva de Los Sabinos, Sótano de la Tinaja, Sótano de Las Piedras, Cueva Chica (El Abra caves) and Cueva del Río Subterráneo (Micos area) (**Fig.4**).

Temperatures were different between caves when considering the entire dataset (mean temperature in Chica: 26.19°C; Pachón: 24.1°C; Sabinos: 23.49°C; Tinaja: 23.35°C; Subterráneo: 23.05°C; Piedras:

21.35°C), with clear tendencies for Chica to be a "hot cave" and for Piedras to be a "cool cave" (**Fig.4A**). Moreover, temperatures were markedly different between different pools in a given cave (**Fig.4A**). Indeed in all caves, the water temperature increased as a function of the depth and/or the distance of the pools from cave entrance (see cave maps in **Supplemental File1**). In all cases, pools located close to cave entrances were always fresher than deeper/more distant pools close to final sumps, with a remarkable gradient of water temperature. In Piedras for example, pool6 (the first large pool encountered at 165m from pit entrance, at a 30m depth) has an average temperature of ~19.7°C, while pool10 (245m from entrance, 50m deep) has an average temperature of 21.9°C and the final pool11 (370m from entrance, 50m deep) has a temperature of 24.6°C. Therefore, there is a 4.9°C difference between these pools of the same Piedras cave, all of which are inhabited by cavefish. The same pattern applies globally to all other studied caves except Pachón: this cave is very small as compared to others, and the so-called main pool and lateral pool form a single body of water that communicates most of the year when water level is high, therefore recording the same temperature in the two pools was expected. Moreover, the pools in the Maryland passage are almost never accessible for measures (**Supplemental File1** and see below).

Average pH values were similar in all studied caves (Chica: 7.48; Pachón: 7.4; Sabinos: 8.01; Tinaja: 7.7; Subterráneo: 7.58; Piedras: 7.8; maximum variation: 0.61), with Sabinos showing the most basic pH values. There was no such pattern as seen with temperature between pools within a cave, except for Piedras (**Fig.4B**). There, pH decreased as a function of depth, with slightly basic values in pool6 (pH=8.11), a pH value of 7.85 in pool10 and a nearly neutral pH in the deep pool11 (7.12).

Finally, average conductivity did not differ between caves (Chica: 631 µS.cm; Pachón: 524 µS.cm; Sabinos: 451 µS.cm; Tinaja: 487 µS.cm; Subterráneo: 444 µS.cm; Piedras: 525 µS.cm; maximum variation: 187 µS.cm), and no marked differences were observed between pools within a cave, except for Tinaja (**Fig.4C**). However, in this case, the variations in water conductivity did not follow the

topography of the cave, as values decreased from pool2 (556 µS.cm) to more superficial pool1 (459 µS.cm) and to the deepest pools 3-4 (359µS.cm).

Our data suggest that the water conditions experienced by *Astyanax mexicanus* cavefish in different caves and especially in different pools within a single cave, can vary significantly. In summary, the cave environment is not homogeneous, and each pool may be considered as a microhabitat (when separated).

Hypoxia, a shared and extreme feature in cave waters

Cavefish have evolved hypoxia resistance through changes in the size or numbers of their erythrocytes, changes in the hypoxia inducible pathway gene regulation, and changes in gill morphology and the number of oxygen sensors (Boggs et al., 2022; Boggs and Gross, 2024; van der Weele and Jeffery, 2022). To better understand the extent of hypoxia levels they face in their natural habitat, we measured oxygen content in the water of several caves in February 2022 and February 2023 (**Fig.5**). As a control, we probed Río Choy (river location #2 on **Fig.1A**) and found 88.1% O2 saturation, corresponding to a dissolved O2 concentration of 7.02mg/L, which is consistent for a running river and favorable for fish spawning and growth (**Fig.5B**). Conversely, in Pachón, Sabinos, Piedras, Chica and Subterráneo caves, we recorded strongly hypoxic conditions (**Fig.5A**).

The most extreme conditions was observed in Chica pool2 with 10% O2 saturation, i.e., 0.88 mg/L dissolved O2, which is in theory too low for fish populations to survive (**Fig.5B**). A simple explanation for this dramatic situation is the presence of a very large colony of bats in this cave, resulting in high levels of decomposing organic matter in the water (bat guano and cadavers, insects, leeches, ostracods…). Yet, Chica is inhabited by a large population of cavefish, which we have previously shown to be well fed, well grown, in good physical condition, and actually the biggest fish we have measured among all caves tested (Blin et al., 2020)(**Fig.5C**).

All other cave pools tested showed low to very low O2 water content as well, all corresponding to lethal or very stressful conditions for "normal" fish (**Fig.5A-C**). Interestingly, in Sabinos where we have recorded O2 two consecutive years (2022-2023), hypoxia was more pronounced in superficial pool1 (mean 42.7% O2 saturation) than in deeper pool2 (mean 70.7% O2 saturation), in a reproducible manner (**Fig.5A**), suggesting another level of heterogeneity between the two pools of this single cave. In this regard, it is noteworthy that the cavefish in Sabinos pool2 are significantly larger than those in pool1 (Blin et al., 2020)(**Fig.5C**). Moreover, the presence of a fresh-water white sponge (Porifera) exclusively in Sabinos pool2 but not pool1 (Legendre et al., 2023a) could be explained by oxygen content as well, in addition to substrate and temperature differences.

Finally, an interesting observation was made in Pachón. The water of the main pool was strongly anoxic in 2022 (2.13mg/L dissolved O2) but less so in 2023 (4.86mg/L dissolved O2). We interpret that this difference must be due to the extremely low water level in Pachón main pool in February 2022, the lowest we have ever witnessed in this cave, with the fish swimming in a very reduced body of still water totally disconnected from the lateral pool (see Legendre et al., 2023b for pictures and see also below, **Fig.8**). This further suggests that cavefish not only have to cope with severe hypoxia, but they also have to cope with fluctuating hypoxia levels over time.

Annual longitudinal measurements of water parameters in caves

Having documented the variations in physicochemical parameters of the water between surface streams and caves, between caves, and between pools within a cave, we next sought to investigate potential variations over the course of the year and the seasons. To this end, we installed and secured different types of probes (see Methods and **Fig.1B**) in five different caves: Pachón, Sabinos, Piedras, Chica (all El Abra caves) and Subterráneo (Micos area). Probes installed in March 2019 were recovered in February 2022, after 3 years, due to Covid travel interruption. Those installed in February 2022 and February 2023 were recovered the next year, in February 2023 and February 2024, respectively. Some

- but not all - of the probes provided full year records. In addition, to help interpreting the data from these probes, we set up camera traps near some cave entrances to better understand the hydrological regime and water flow into and out of the caves. This was done for Chica and Pachón (and Subterráneo, but unfortunately there the camera disappeared).

Cueva de Los Sabinos

In Sabinos, we obtained records in pool 1 and pool2, in 2019 and 2022-23, for temperature, conductivity and pH, including some in duplicates (**Fig.6**). The average yearly temperature was 22.9°C in pool1 and 24.3°C in pool2 (var. 1.4) in 2019, and 24.0°C in pool1 and 24.3 in pool2 in 2022-23 (var. 0.3). The 2019 values are very close to one-off measurements shown above (22.5°C and 24.4°C, n= 16 and n=18 point measures in February and March, respectively), and they confirm the temperature gradient according to depth in the cave. Moreover, for both recorded years, the temperature in pool2 was very stable (turquoise curve/2019: min 24.24°C; max 24.36°C; var. 0.13; light blue curve/2022: min 24.19°C; max 24.49°C; var. 0.30). Conversely, mild or more important and sudden temperature fluctuations were observed in pool1, both in 2019 (dark blue curve, min 22.35°C; max 23.29°C; var. 0.94) and 2022-2023 (blue curve, min 22.7°C; max 25.6°C; var. 2.9). In addition, as observed from the 2022 recordings, pH varied inversely with temperature in pool1, and conductivity varied globally in parallel with temperature (pink curve, min 459 µS.cm; max 637 µS.cm; var. 178) (**Fig.6**). We interpret these data as pool1 being more exposed to the direct influence of rainy days and flooding than pool2, and having a more variable hydrological regime. Thus, after a gradual increase that started in March-April 2022, i.e. the beginning of the rainy season (also seen on the 2019 curve), four sudden peaks of temperature increase occurred in August and September 2022 (16 August and 6, 20, 27 September), synchronous with decreases in conductivity (red curve), indicating rainwater entering the cave.

Very interestingly, on 16 August 2022, when a massive and sudden influx of water flooded the cave as deduced from the 1.39°C temperature increase observed on a single day in pool1 (blue curve), a

smaller but significant and synchronous peak (+0.2°C) occurred the same day in pool2 (green curve, **Fig.6**). These data strongly suggest that the flood of that day penetrated to the bottom of the cave, and that pool1 and pool2 became connected at least during a few days. These results have important outcomes for Sabinos cavefish biology (and other inhabitants of the cave, arthropods, bats and sponges (Elliott, 2018; Legendre et al., 2023a)). First, they are sporadically exposed to catastrophic and stressful events of environmental change, to which they must react and adapt. Second, the cavefish populations of pool1 and pool2 are mixed from time to time, which means that genetic differentiation cannot occur and the Sabinos cavefish must be genetically homogeneous.

Cueva del Río Subterráneo and Cueva Chica

Subterráneo and Chica are two caves in which introgression of surface fish swimming in surface streams occurs regularly, thus they host hybrid fish populations.

In Subterráneo, surface fish are washed into the cave entrance at the rainy season, as witnessed by their abundance in the first perched pool of this cave, and their presence further down in the cave, where they co-habit and breed with cavefish. Others epigean species of Cichlidae and Poeciliidae as well as crayfish are regularly observed in the cave. We have personally observed the violent flooding of this cave, so seasonal variations in water parameters were expected. There, in pool2 we obtained one year of records of temperature (in triplicate), pH and conductivity (Feb2022-Feb2023) (**Fig.7A**). The temperature curves recorded in triplicate with three different types of instruments (different blue shades on **Fig.7A**) showed identical profiles, suggesting that the data we present in this paper are consistent and reliable. As anticipated, over the year, Subterráneo pool2 was subjected to strong, sudden and synchronous variations in temperature (min 19.8°C; max 28.2°C; var. 8.4), pH (pink curve, min 7.15; max 8.64; var. 1.49) and conductivity (red curve). Note that we consider the drop in conductivity to almost zero in August 2022 to be an artifact of unknown origin (the probe probably got transiently out of the water). After a gradual increase in temperature between February and August,

several drastic increases peaked on 17 August, 5 and 8 September and later, and an unexpected rapid drop to ~20.88°C occurred in December (**Fig.7A**). While the increases in water temperature in August and September are consistent with the idea of water flowing into the cave from the surface after heavy rains, the winter decrease in pool2 temperature may indicate that fresher water from the underground aquifer entered the cave, possibly by inflow from the final sump. Alternatively, cooler air circulation may be more intense during this period. Overall, the massive variations in temperature and other parameters observed in Subterráneo suggest a drastic, complex and dynamic hydrological regime to which the resident cavefish must be exposed.

In Chica, it is believed that surface fish from the nearby Río Tampaón enter the cave through the deepest part of the cave, rather than through the entrance used by humans at ground level. In this cave, in pool2.5 (a medium size pool between pool2 and pool3) we obtained one year of records of temperature (in triplicate, identical curves in blue shades), pH (pink) and conductivity (red) (Feb 2022-Feb2023) (**Fig.7B**). The longitudinal measurements confirm that Chica is the warmest cave in the area (average 27.4°C). They also show that the annual variations are extremely important for the three parameters recorded, especially for conductivity and temperature (temperature: min 23.6°C, max 28.1°C, var. 4.5; pH: min 7.07, max 7.95, var. 0.88; conductivity: min 252 µS.cm, max 1560 µS.cm, var. 1307). In Chica like in other caves described above, the temperature increased from February to August (**Fig.7B**). However, contrarily to other caves, this gradual increase of temperature was associated to large peaks and oscillations in water conductivity and pH rather than a regular increase, suggesting a chaotic hydrological regime all year long. Moreover, the fact that the conductivity curve shows several peaks (not drops) strongly suggests that the water entering the cave is of river origin, most probably flowing up the cave from its depth from the Río Tampaón. Then, from the end of August, the temperature oscillated and underwent several sudden, transient and significant decreases (17 Aug, 5/7/16/20 Sept, 1/5 Dec), perfectly synchronized with transient decreases in conductivity indicating rainwater entrance. Such events of Chica cave flooding from the dry “arroyo” at its entrance was not clear from discussions with the locals. So we set up a time-lapse camera focused on the cave entrance

and obtained a year's record of rainfall and outside air temperature, from February 2023 to February 2024 (**Fig. 7C**). Rainy days and the resulting water regime were easy to identify and interpret (**Fig. 7CD**). Heavy rains caused the arroyo to flow actively and rainwater to enter the Chica Cave on a massive scale four times in 2023 (twice in May, twice in October-November), confirming the hypothesis that water can enter in Chica by both ends, its entrance (from rainfall) and its final sump (from Río Tampaón). Taken together, these data suggest that Chica cavefish are exposed to the most extreme and changing hydrological regime of any *Astyanax* cave we have explored.

Cueva de El Pachón

Pachón is the most studied *Astyanax* cave, including for field research (Blin et al., 2020; Espinasa et al., 2017; Espinasa et al., 2023; Hyacinthe et al., 2019; Krishnan et al., 2020; Legendre et al., 2023b; Ornelas-Garcia et al., 2018; Peuss et al., 2020; Simon et al., 2017), so understanding its ecology is paramount.

In 2019, we recorded temperature in the Pachón main and lateral pools. In sharp contrast to other caves, water temperature was very stable (main pool/4months, min 24.4°C, max 25°C, var. 0.6; lateral pool/9months, min 24°C, max 25.4°C, var. 1.4) (**Fig. S3**) and showed only a minor and continuous increase between March and September. The first months of the 2022 records confirmed this trend (**Fig. 8A**, blue curve). We propose that these almost negligible variations in water temperature follow the evolution of the cave air temperature (red curve on **Fig. 8A**, recorded in 2022-2023: min 23.9°C, max 25.1°C, var. 1.2), which in turn is influenced, albeit strongly buffered, by seasonal variations in outside air temperature (**Fig. 8D**, green curve). This scenario is consistent with Pachón being a perched cave that is not flooded by rivers that overflow during the rainy season, and that receives water mainly by percolation and/or inflow from the aquifer through the depth of the cave.

In February 2022, when water was extremely low in the cave as mentioned above, we setup a multi-parametric probe at the back of the main pool (see map in **Supplemental File1**). Water level was

included in the recorded parameters (dark blue curve on **Fig. 8A**). To our surprise, between March and May 2022, the water level continued to fall by another meter, with very regular oscillations that we are tempted to interpret as pumping episodes through the pipe installed there. For the cavefish population swimming there, the amount of water available must have been minimal and the situation extremely dangerous for at least two months. After this “catastrophic” event, the water level rose by more than 4 meters in about 4 months, returning to the "normal" water level that we and others have repeatedly observed when visiting the cave at different seasons (including when we recovered the multi-parametric probe in February 2023). This shows that Pachón main pool occasionally undergoes variations of hundreds to thousands of cubic meters of water volume. Before 2022, the last known record of such low water in Pachón was in March 2003, when Jeffery, Yamamoto and Espinasa took this opportunity to discover and survey the Maryland extension galleries.

Unfortunately, one consequence of the extremely low water levels is that the temperature records cannot be considered reliable after ~mid-May 2022 (blue curve, shaded in **Fig. 8A**), because the multi-parametric probe is likely to have been partially dried/damaged. Likewise, the two peaks observed simultaneously on the conductivity, resistivity, ammoniac and nitrate curves are likely artefacts due to very low water levels. However, the available records of pH, conductivity and resistivity (which mirror each other hence are considered as reliable), as well as nitrate and ammonia, suggest that water conditions in Pachón were relatively stable throughout the year, despite the large amount of water that replenished the water chamber.

Finally, locals caught our attention on strong episodes of water resurgences at the bottom of the dry arroyo under the Pachón cave (**Fig. 8B**). This suggested that when water level is high, the cave might discharge into the arroyo *via* this resurgence (at least partially). We installed a time-lapse camera to capture the rainfall and the water regime in this arroyo from February 2022 to February 2023 (**Fig. 8CD**). The data collected show numerous rainy days in September 2022 (orange on **Fig. 8D**), associated with a period of about a month during which water flowed in the arroyo (grey on **Fig. 8D**), fed by the

resurgence (Fig. 8CD). Strikingly, this period coincided with the time when there was an abrupt raise of about 1.5 meter in water level in the Pachón cave pool (Fig. 8D, dark blue curve superimposed). We interpret these data as the heavy rain contributing to cave pool recharge (probably more through percolation than from the underground aquifer), followed by cave pool overflow into karst cavities and network connected to the resurgence at the level of the arroyo (Fig. 8B). It means that the water level in Pachón cannot be higher than a certain threshold (about 4 meters above the level of our multi-parametric probe) or it will overflow. It also means that some cavefish may be washed out of the cave during these episodes, further contributing to the decline of the population in this cave (Legendre et al., 2023b).

Discussion

The ecology, hydrodynamics and classification of groundwater ecosystems is a subject of research and attention (Aquilina et al., 2023; Robertson et al., 2023), especially in the interest of groundwater ecosystem functions and services (Hose et al., 2023; Kretschmer et al., 2023). This report is, to our knowledge, the first to document and interpret cave water quality and hydrodynamics for an integrated view of cave biology and the evolution of cave organisms (Malard et al., 2023; Torres-Paz et al., 2018). The point and longitudinal measurements of water parameters that we obtained in several caves and rivers hosting populations of *Astyanax mexicanus* between 2009 and 2024 have profound implications for understanding the evolution of cavefishes and the biology of the species in general.

Macrohabitat

The surface fish *Astyanax mexicanus* swim in subtropical freshwaters in Mexico and as far north as Texas. Conversely, the cave-adapted morphs of the species inhabit a series of 33 caves in a restricted

region of North-East Mexico (Elliott, 2018; Espinasa et al., 2018; Espinasa et al., 2020; Mitchell et al., 1977). Water quality sampling in this area, in surface rivers, ponds, springs on the one hand and in caves on the other, shows marked differences between epigean and hypogean waters and reveals strong signatures of these two macrohabitats: cave water is fresher, much less conductive than surface water, and highly anoxic. Our large-scale dataset corroborate earlier punctual and sparse reports on these variables (Breder, 1942; Elipot et al., 2014b; Ornelas-Garcia et al., 2018; Tabin et al., 2018).

Darkness and the absence of primary production are generally considered the hallmarks of the cave environment. However, water quality may also play a very important role as an environmental variable driving the evolution and adaptation of cavefish, and as a *bona fide* contributor to this so-called "extreme" habitat. In fact, the large shift in water conductivity from surface to cave has been proposed as a potential abiotic stress factor that could have triggered an HSP90-related stress response and unmasked cryptic variation, allowing the initial evolution of eye loss during cave colonization (Jarosz and Lindquist, 2010; Rohner et al., 2013). To our knowledge, low levels of dissolved oxygen have not been tested in this direction, but it is hard to imagine that the severe and sudden hypoxia encountered by the first cave dwellers did not have an effect on their stress response and adaptation.

Astyanax mexicanus belongs to the order Characiformes and the family Characidae. Yet, before evolving the specific hypoxia-resistant traits that extant cavefish possess today (Boggs et al., 2022; Boggs and Gross, 2024; van der Weele and Jeffery, 2022), they must have survived in conditions that are lethal to highly stressful for most fish. This may indicate that *Astyanax mexicanus*, as a species, has hidden characteristics that enable it to withstand such harsh conditions. If true, such a preadaptation to hypoxia, together with other traits like the ability to breed in the dark (Simon et al., 2019), would also explain why only *Astyanax* has colonized and thrived in Mexican caves. In fact, fish from others families like Poeciliidae and Cichlidae that are occasionally observed in these same caves (regularly at least in Chica and Subterráneo) are always in poor condition, they do not survive and do not form established populations in the *Astyanax* caves network (personal observations). An alternative

possibility is that, when surface fish colonized caves around 20.000 years ago (Fumey et al., 2018; Policarpo et al., 2021), cave water was more highly oxygenated and they did not have to cope with anoxia on top of darkness. Notably, in addition to the oxygen variable, we also found surface fish swimming in water with a pH of 9.7, suggesting that the extant species (and ancestor) as a whole are quite resilient and plastic to environmental water conditions.

Mesohabitat

Every cave is special and unique. Besides the low conductivity of the subterranean waters that is a shared feature of all caves studied, other parameters can vary significantly between locations. One of the most important may be the temperature of the water. The difference between Chica, the hottest cave at ~27-28°C, and Arroyo, the coolest at ~16-17°C, is huge and must have important consequences for reproduction and development, metabolism, energy homeostasis and behavior of the cavefish populations – as they are poikilothermic, i.e. their body temperature varies with the ambient temperature eg (Zahangir et al., 2022). The origin of such differences probably lies in the general geological and biological characteristics of the caves. In Chica, for example, the tens of thousands of bats that live there act as a powerful air heater, while the entrance to the very large and long Arroyo Cave is located at the bottom of a 25-meter pit embedded in the coolness of the forest. Many other combinations are possible, and air dynamics linked to cave geomorphology may have a certain influence.

Interestingly, river-dwelling *Astyanax* and cavefish from the Tinaja and Molino caves have a temperature preference that is significantly higher (28.3°C) than Pachón cavefish (25°C), as measured in behavioral assays (Tabin et al., 2018). The difference between surface and cave fits well with the fact that temperatures in rivers are significantly higher than in caves. The difference between caves is consistent with our dataset and suggests that local adaptations have occurred in different cave populations in response to specific environmental water cues. It would be extremely interesting to

address the genetic determinism of the physiological and behavioral adaptations present in, e.g., the Chica and the Arroyo populations discussed above, see also (Tabin et al., 2018). In the same vein, it would be important to study the genetic basis of hypoxia resistance, as the physiological changes needed to survive in water with 10% O2 saturation (Chica) are likely to be different from those needed to survive in water with more than 60% O2 saturation or fluctuating O2 levels (Pachón, Sabinos). Moreover, water parameters and particularly oxygen levels - rather than the cave vs surface environment - seem to influence *Astyanax* gut microbiome, which has essential roles in the regulation of metabolism, physiology and health (Ornelas-Garcia et al., 2018).

Another aspect that makes each cave different is the variety of seasonal and multi-annual hydrological regimes. From our longitudinal multiparametric measurements, it appears that water quality can be either stable (Pachón) or vary slightly or more significantly throughout the year, either gradually or suddenly (other caves). Some caves receive water mainly from outside precipitation (Sabinos, Subterráneo), or from percolation and upflow from the aquifer (Pachón), or from both precipitation and upflow from the nearby river (Chica). Some caves are perched and drain into the hydrological system, so their water level is limited (Pachón), while others can be completely flooded. In addition, in the caves where a pipe is installed such as Pachón and Chica, the water level and the amount of water available may in principle be subject to very strong fluctuations due to anthropic activities, which we did observe in both cases (very low levels in Pachón in 2022 and Chica in 2024). Thus, qualitatively and quantitatively, the hydrodynamics of the studied caves are different - although globally the discharge and the recharge are very fast, because karstic systems are flashy by nature (Aquilina et al., 2023).

Incidentally, caves that are flooded after rainfall are likely to receive large amounts of phytosanitary chemicals from nearby sugar cane plantations (e.g. Subterráneo), a situation that is detrimental to cave fish and other cave animals, as well as to the quality of the underground water. Our qualitative observations seem to suggest that fish from Subterráneo cave are more infested by parasites than in other caves. In principle, caves that are not flooded escape this threat (e.g. Pachón), although

contaminated water may also enter through upflow from the aquifer. To our knowledge, this has not been studied in *Astyanax* caves, but we expect it to be an important conservation issue (Boulton et al., 2023; Hose et al., 2023).

Such complex and specific hydrodynamics as we have described make the cave environment less stable and homogeneous than commonly thought, at least from the perspective of cavefish. Researchers who enter caves for fieldwork usually do so at the end of the dry season for obvious safety reasons, so they have a biased view of what cave life is. In fact, cavefish experience daily (driven by the entry and exit of bats at dusk and dawn (Beale et al., 2013)), seasonal (driven by rainfall and regional climate) and other non-natural irregular rhythms (driven by human activity such as pumping of water, mining activities…). Different caves are differently impacted by these three types of influences, depending on their configuration and geology (Elliott, 2016; Elliott, 2018). Hence, each cave environment is unique, and each cave population has evolved according to different selection pressures (besides the critical and shared absence of light). Although the causal relationships between specific environments and local cavefish genetics will probably always remain elusive, the different conditions found in different caves may have contributed to shaping slightly different evolutionary trajectories (Borowsky, 2008; Policarpo et al., 2024).

Another consequence of our findings is the need for cavefish to cope with stress associated to fluctuating environment (Cobham and Rohner, 2024). Some authors have described reduced behavioral stress responses in cavefish populations compared to surface fish with a novel tank test (Chin et al., 2018), and they hypothesized that this corresponds to the transition between predator-rich epigean and predator-free cave environments. However, according to the present data, we would argue that the environment of the cave is not free of stress. On the other hand, others have described a mutation in the *MAO* gene (Elipot et al., 2014a), which is distributed in all the cavefish populations of the El Abra region, and which confers very low basal anxiety to fish in stable conditions but also strongly increases the stressability of fish in response to a novel tank (Pierre et al., 2020). These results

are consistent with cave life being predator-free and rather stable most of the year, as well as with the need to react fast and efficiently to sudden and sometimes cataclysmic events that occur during the rainy season. As year-long records of cavefish behaviors are technically impossible to obtain in most caves, indirect monitoring of their activity and distribution in the caves through underwater long-term passive acoustic recordings would bring some valuable insights on how they cope and react to seasonal changes in each cave (Aalbers and Sepulveda, 2012; Lammers et al., 2008).

Microhabitat

Finally, our data suggest that within a cave, each pool is different. Again, the main variable is temperature, which shows a low-to-high gradient from more superficial pools to pools further away from cave entrance, in a very reproducible manner in all caves. Differences can be important, such as ~5°C between pool6 and pool11 in Piedras, or ~7.5°C between Subterráneo pools. As cavefish seem to have temperature preference that are different between caves and that this trait is genetically encoded (Tabin et al., 2018), our findings raise the question of how this temperature preference is established and regulated at cave population level. In Piedras, there is also a high-to-low pH progression with depth, and in Tinaja different pools have different conductivities.

In addition to these variable water parameters, we have also observed that different pools within a cave vary by other biotic and abiotic aspects. For example, Sabinos pool1 -but not pool2- is located under a bat roost, so food guano is available; conversely Sabinos pool2 -but not pool1- contains freshwater sponges (Legendre et al., 2023a), so the chemical signature of the water including numerous sponge metabolites must be very specific. As another example, some pools have a muddy substrate (Tinaja pool2) while others have a rocky substrate (Tinaja pool1), thus offering different compositions in microorganisms.

Our analyses further show that whereas cave pools are separated when researchers visit them, they connect from time to time when water level raises. In Pachón, fish residents of main and lateral pool

may be separated for several months, but they will eventually mix, at least once a year, when water level raises. In Sabinos too, where pool1 and pool2 are distant from about 150 meters and at distinct depth, mixing occurs after heavy rain and flooding. This is consistent with the lack of genetic differentiation between pools within caves (unpublished results). Moreover, such mixing may result in the redistribution of fish individuals and populations between pools from a year or a month to another, again generating stress, and forcing them to re-adapt and re-explore their environment to map their novel swimming space - a task achieved without vision, as always.

Conclusion

Cave colonization by surface *Astyanax* occurred about 20,000 years ago, twice independently in the Micos area on the one hand and in the El Abra/Guatemala region on the other (Policarpo et al., 2024). At that time, the biotic environment, the geography, the geology/topography, the climate, the hydrodynamics and the water quality must have been different from today. In any case, the greatest challenge for the first cavefishes was the absence of light. However, in the course of their progressive colonization of karstic cavities in a “stepping stone” mode (33 known today), they have encountered different, specific, sometimes extreme conditions in water regimes and qualities that have undoubtedly shaped their evolutionary trajectories.

Methods

Geographical area studied

The stations studied are located in the San Luis Potosi and Tamaulipas regions of northeastern Mexico (part of the Sierra Madre Oriental). In this area, approximately 130 km long and 60 km wide, between 2009 and 2024 we collected data on water physico-chemical parameters from 13 caves and 27 surface stations (**Fig.1A**). All cave stations were inhabited by *Astyanax* cavefish (or hybrids), and all surface stations were inhabited by *Astyanax* surface fish (and usually other species as well). Some stations were surveyed many times, with different interval times (days, months, years). The 13 cave stations include a total of 183 measurement points, and the 27 surface stations include a total of 77 measurement points (**Supplemental Table1**, raw data). In addition, longitudinal recordings were performed in a subset of cave stations.

Recording instruments

We used different methods and apparatuses to record water parameters (**Fig.1B**).

Point measurements.

One-off measurements were taken using the Combo Hanna HI98129 meter (Hanna Instruments, USA) for pH, electrical conductivity and temperature. The Combo was calibrated regularly in the laboratory and in the field using the HI98129 manufacturer's protocol.

Some additional pH controls were performed with colored Tetratest (TETRA, Copyright © Spectrum Brands, Inc., USA, and JBL GmbH & Co., Germany). All colored test used were recently purchased and were open only for the fieldtrip.

A field oximeter Hanna HI98193 (Hanna Instruments, USA) wired with HI764073 probe was used to measure dissolved oxygen.

Longitudinal measurements.

For long-term measurements, in most caves we used HOBO probes. Water or air temperature measurements of 2019, 2022, and 2023 were made by MX2201 and MX2202, pH/temperature by MX2501, and electrical conductivity/temperature by U24-001 (Onset HOBO® Data Loggers, USA).

A multi-parametric probe AQUATROLL 600 (type 74050, IN-SITU©2022 In-Situ Inc., USA) was used for longitudinal measurements in the Pachón cave (as well as for some punctual measure in February 2022 in other cave). This probe is designed with four smart sensor ports for interchangeable sensors, an LCD screen and Bluetooth connectivity. For this study, it was fitted with four sensors as follows: one for pH/ORP (758768), one for Nitrate NO3-N (ISE, 742828), one for Ammonium NH4+N (ISE, 704780) and one for electrical conductivity/temperature (778296). Details on probes resolution, accuracy, range and calibration solutions are available in the manufacturer's instructions and technical sheets.

To configure the probes and retrieve data, we used a mobile Bluetooth connection with mobile applications designed specifically for these probes. The VuSitu app (In-Situ, Inc., version 1.19.12) was used for AQUATROLL 600. The HOBOmobile app (ONSET, HOBOmobile® for Android, version 2.0 edition: 1029 ©2019 Onset Computer Corporation) was used for the HOBO MX2201, MX2202, and MX2501 probes. The HOBOware software (ONSET, HOBOware®, version 3.7.19, ©2002-2020 Onset Computer Corporation) was used for the U24-001 probe, which requires an optical connection through the Base-U-4 Optic USB Base Station (Onset HOBO® Data Loggers, USA) to restore or configure the probe. Probes were programmed, configured, and verified in the field just prior to deployment. The majority of probes have logging rates ranging from 1 second to 18 hours. We choose 12 hours for longitudinal studies.

Finally, to record time-lapse photographs of rainy/sunny days and outside air temperature, we used TimelapseCam Pro (Wingscape, WCT-00126).

Probe installation and fixation

Fixing the longitudinal probes was a challenge. The probes had to be small, light, and easy to attach without any prior information about the water flow and the potential debris (rocks, stones, wood...) carried by the water flow. In 2019, five HOBO MX2201 for water temperature were installed in three different caves (Subterráneo pool2, Pachón main and lateral pool, Sabinos pool1 and pool2). We used thin cord (type Cordelette 03 by BEAL, France) to attach loggers to natural reliefs, roughness or holes present on cave walls while preserving the stone and cave integrity. We used nodes to block the cord to prevent the probe from slipping. We attached a small rock to the end of the cable to keep the probe vertical on the water column. We took care to place the probe above the substrate to prevent mud deposition, choosing a permanent piece of water (present in the dry season) in order to reduce the risk of air exposure.

After the success of these test installations, we followed the same procedure in the following years, while further securing the probes fixation. Where natural reliefs were inaccessible, we used an electric drill to make a hole for a dowel and we used a stainless steel anti-theft cable (for computers) to reinforce the system. In addition, larger probes were protected by PVC drainpipe (with holes to facilitate water circulation) (**Fig.1B**).

Statistics and graphical representations

Data analysis and graphs were generated R software environment for statistical computing and some graphics (R Core Team, 2024) and with Excel.

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Author's Contributions

L.L. and S.R. designed the study; L.L. and S.P. performed the field measurement with the help of L.E. and S.R.; L.L. and S.R. analyzed the results; SR and L.L. wrote the article.

Disclosure Statement

No competing financial interests exist.

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Figures and Legends

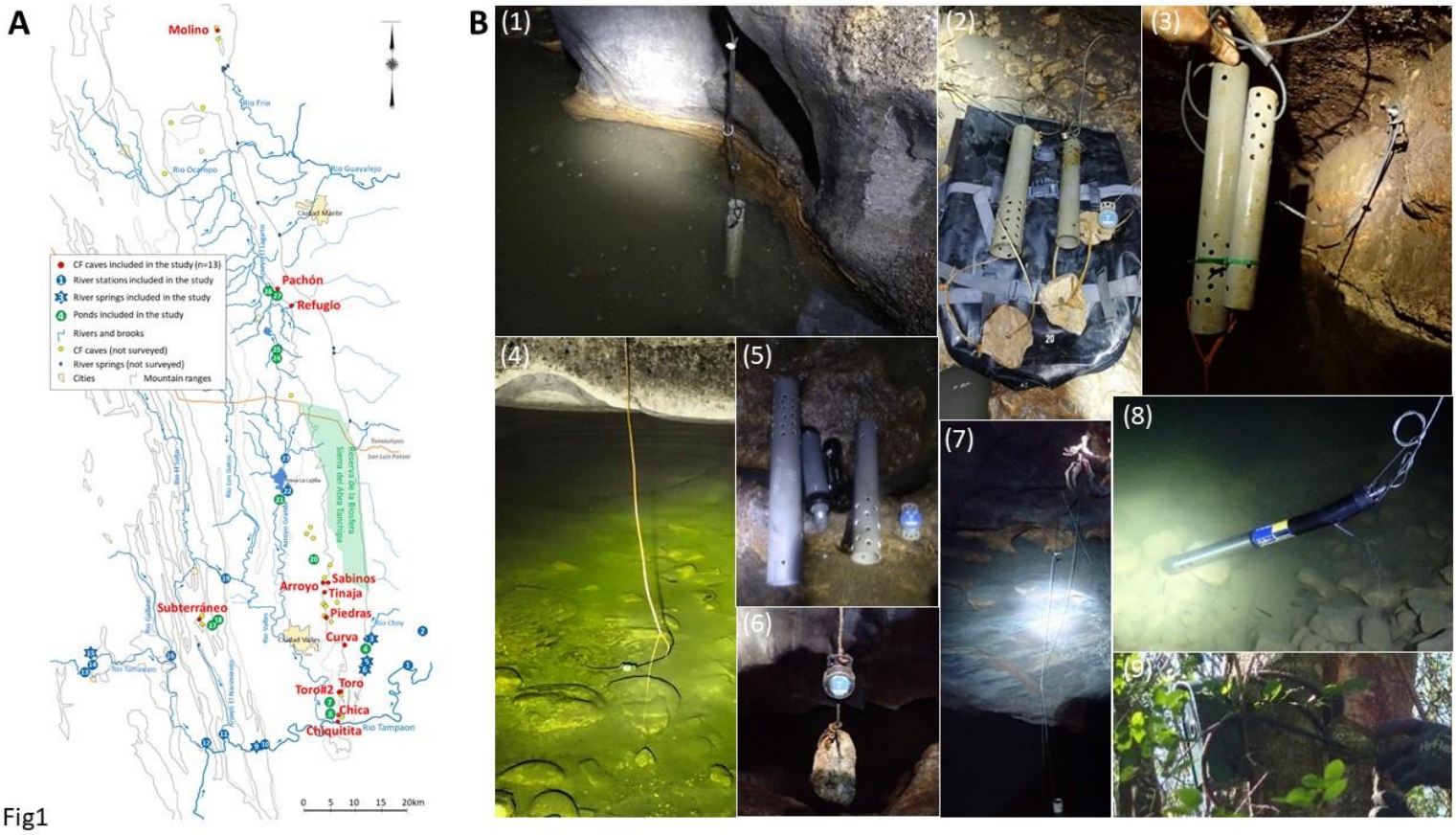


Fig1

Figure 1: Water sampling stations and equipment used.

A: Map of the area. See inset for symbols and legends.

B: Examples of probes used and their installation on sites. (1) Hobo probes (temperature, pH, conductivity) protected in plastic tubes in Chica pool 2.5. (2) Recovery of probes and their protection and attachment devices in Subterráneo. (3) Fixation and securing of two probes in Sabinos pool1. (4) A small Hobo probe installed in shallow water in Sabinos pool2. (5) Preparation of pH and conductivity probes (Hobo) and their protection tubes in Chica. (6) State of conservation of a small Hobo probe after 3 years (2019-2022) in Sabinos pool1. (7) Fixation and securing of the multiparametric probe (In-Situ) in the back of Pachón main pool. (8) Point measurement with multiparametric probe in Subterráneo pool2. (9) Fixation and securing of camera trap for time-lapse recording of water regime at the entrance of Chica. All photos were taken by LL and SR. See also **Supplemental Figure 1**.

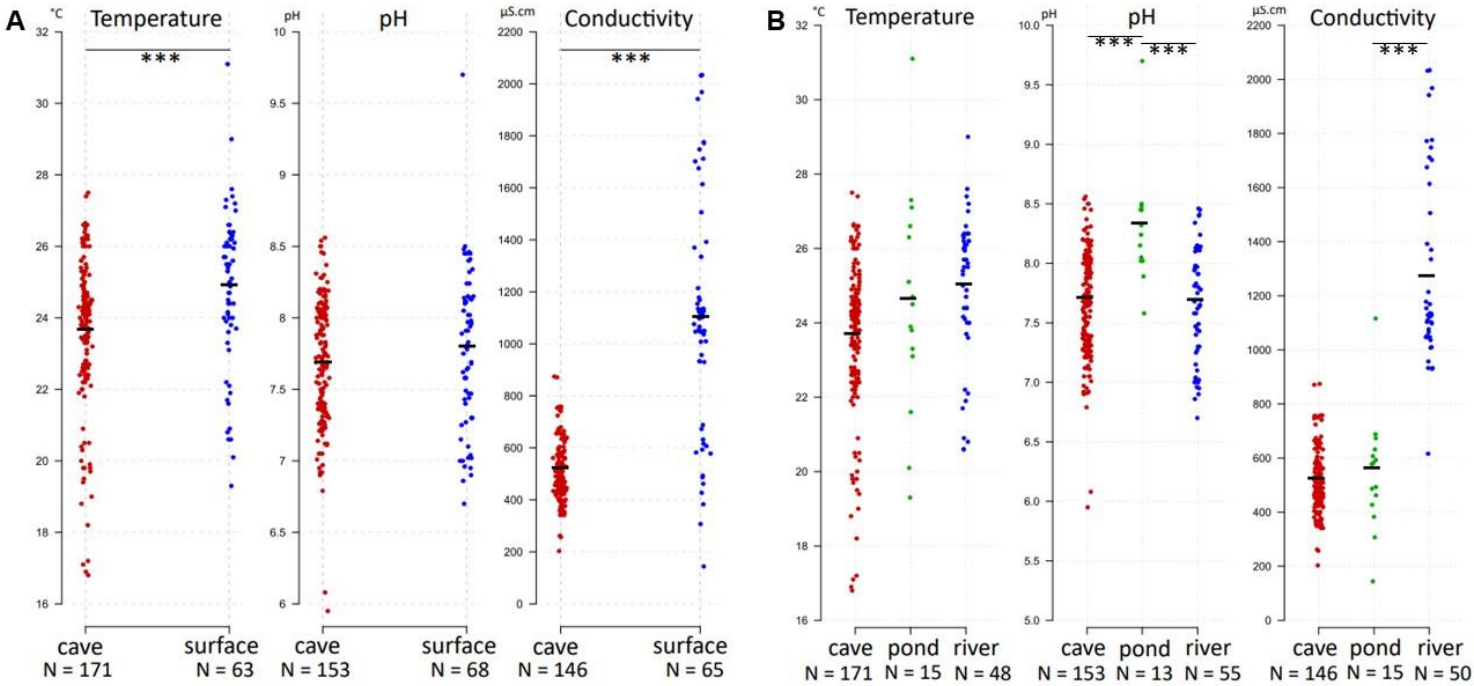


Fig2

Figure 2: Comparing physico-chemical parameters of the water from point measurements in surface waters and in underground caves

A: distribution and comparison of temperature, pH and conductivity between cave (red dots) and surface waters (blue dots).

B: distribution and comparison of temperature, pH and conductivity between cave waters (red dots), still surface water (green dots), and running surface waters (blue dots).

Wilcoxon - Mann Whitney tests (see **Table1**).

Table 1: Summary statistics for point measurements of water physico-chemical parameters in caves, and in still and running surface waters (2009-2024).

a, comparison between cave and surface;

b, comparison between running and still surface water.

Wilcoxon - Mann Whitney tests.

	Sampling dates	Temperature (°C)				pH				Conductivity (µS.cm)			
	2009-2024	mean	median	min	max	mean	median	min	max	mean	median	min	max
Caves, all	2009-2024	23.59 N=171	24.06	16.8	27.5	7.67 N=153	7.69	5.95	8.56	508 N=146	490	203	874
Surface, all	2009-2024	24.86 N=64	25.3	19.3	31.1	7.78 N=68	7.82	6.7	9.7	1092 N=65	1098	144	2034
a		**p=1.8e-5**				**p=0.18 (NS)**				**p=2.5e-20**			
Surface, running	2009-2024	24.92 N=48	25.5	20.6	31.1	7.65 N=55	7.66	6.7	8.46	1256 N=50	1127	616	2034
Surface, still	2009-2024	24.66 N=15	24.5	19.3	29	8.32 N=13	8.24	7.58	9.7	545 N=15	578	144	1116
b		**p=0.68 (NS)**				**p=0.00019**				**p=5.2e-8**			

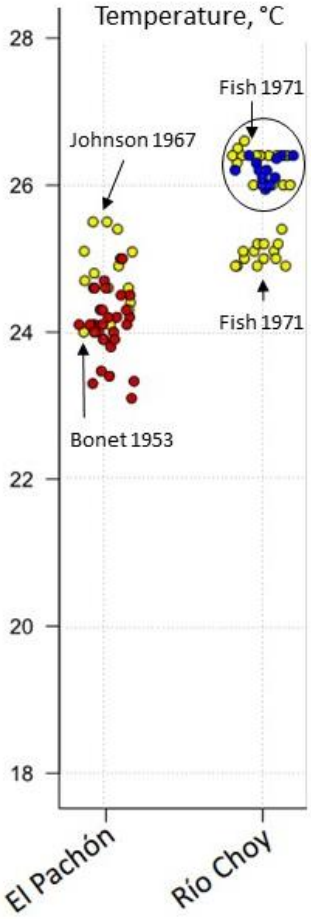


Fig3

Figure 3: Stability of water temperature measurements over 7 decades (1953-2024)

Red dots (Pachón cave) and blue dots (river, Nacimiento del Río Choy) are from our dataset. Yellow dots are from ancient literature, with the author's name & date.

For the surface station Nacimiento del Río Choy (blue dots; river location #3 on **Fig.1A**), a black circle shows a group of ours & old data (Fish, 2004) close to 26°C. Another group of only older data is close to 25°C, also from Fish (2004). Fish showed that temperature could vary within days with flooding (e.g., between 5 June and 14 August 1971) at this location, with also massive variations in discharge, turbidity and water chemistry.

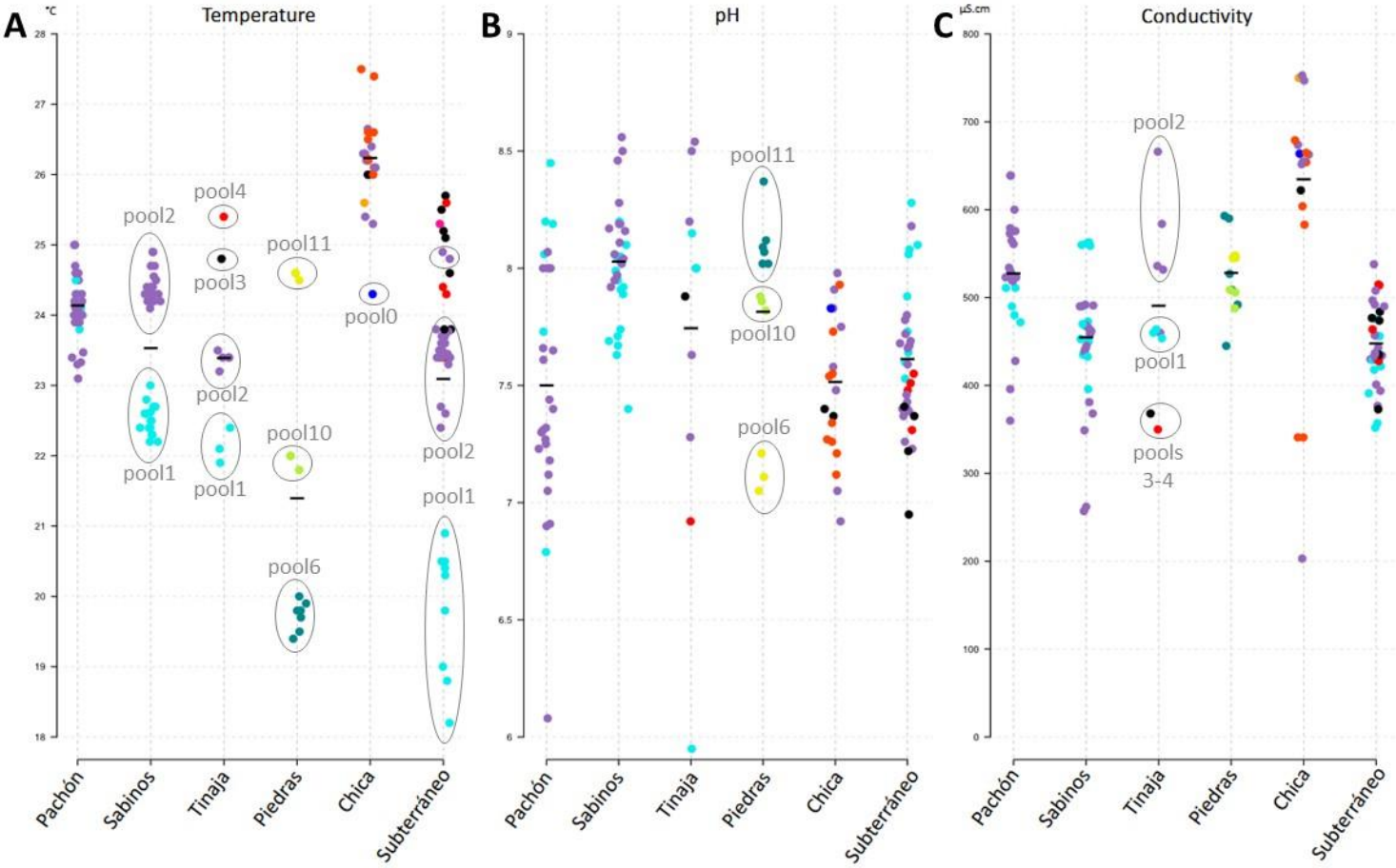


Fig4

Figure 4: Comparing physico-chemical parameters of the water between different caves, and between different pools in a single cave

A: Temperature

B: pH

C: Conductivity

In the 3 graphs, in each cave, dots are color-coded according to the pool in which measurements were made. When useful, pool numbers are indicated, with number 1 being closest to the cave entrance, and higher numbers corresponding to successive pools encountered, further away from entrance and/or deeper in the cave.

A		Feb 2022		Feb 2023	
		% saturation	C (mg/L)	% saturation	C (mg/L)
Pachón	Main pool	25.5 *	2.13 *	60.2 *	4.86 *
	Lateral pool	65.7	5.37		
Sabinos	Pool 1	42.6 *	3.66 *	42.8	3.72
	Pool 2	72.2 *	5.89 *	69.2	5.7
Piedras	Pool 6	51.3	4.61		
Chica	Pool 2	10.9 *	0.88 *		
Subterraneo	Pool 2a			28.4	2.38
	Pool 2b			38.5	3.18
	Pool 3			34.3	2.73
	Pool 3.5	36.9	3.00	45.2	3.63
	Pool 4			44.4	3.45
Rio Choy		88.1 *	7.02 *		

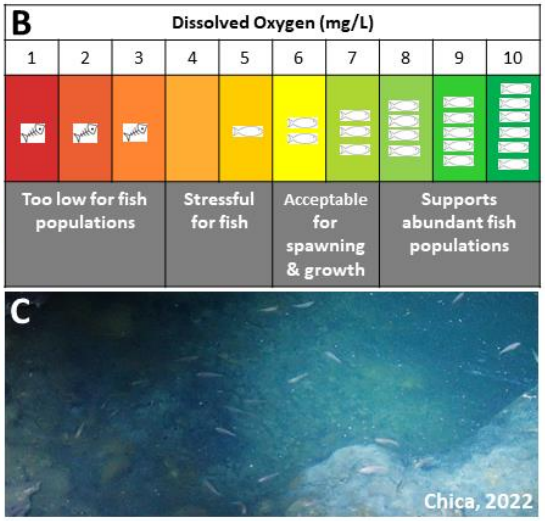


Fig5

Figure 5: Oxygen content in caves and rivers.

A: Data are from single measurements, except those with an asterisk (*), which are the mean value of 3 measurements made at 5 minutes intervals.

B: Key color code for toxicity, adapted from Wikipedia.

C: Picture of healthy-looking cavefish swimming in Chica, a strongly hypoxic cave.

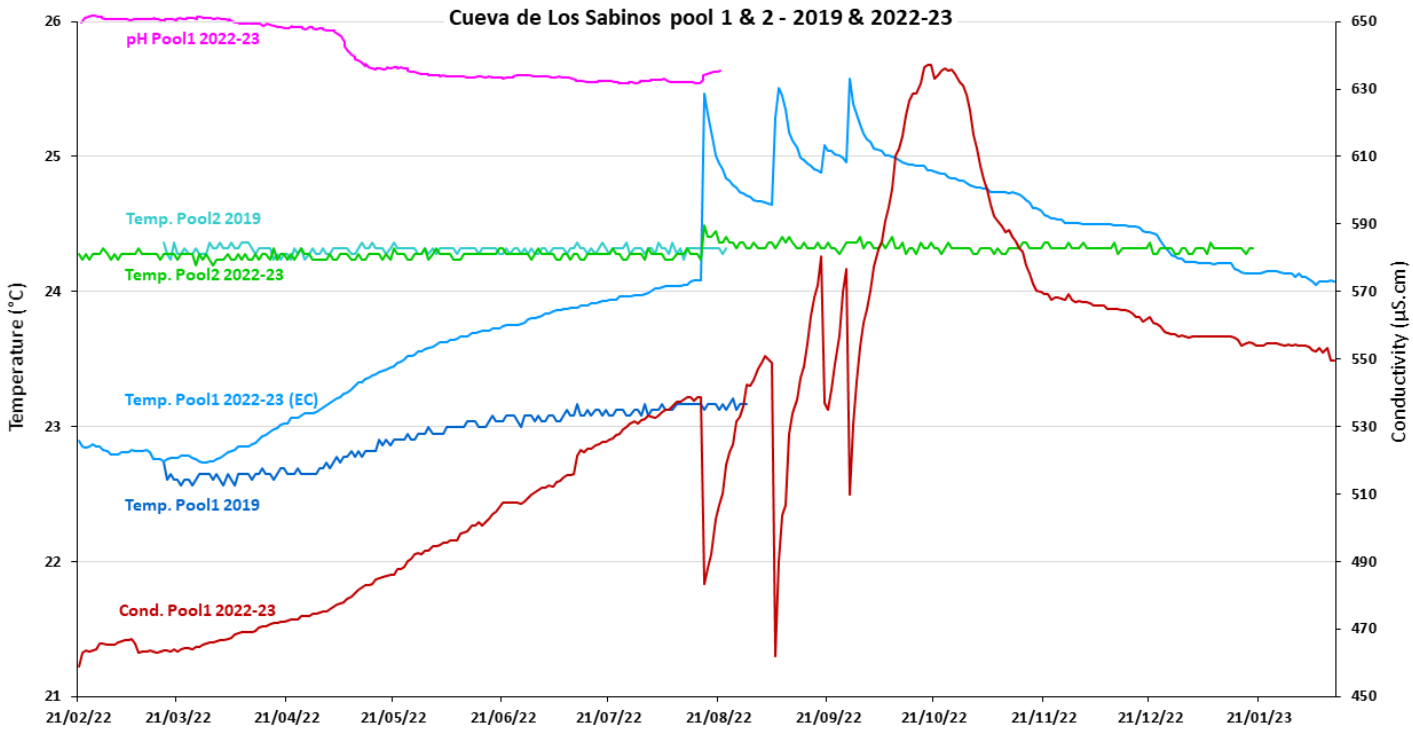


Fig6

Figure 6: longitudinal measurements of water parameters in Sabinos

The evolution of temperature, pH and conductivity, along the year 2019 and from February 2022 to February 2023 in Sabinos cave pools 1 and 2. Color codes for recorded parameter, pool and year of interest are indicated. The temperature in Pool1, year 2022-23, was recorded in duplicate from the conductivity probe (EC, shown) and the pH probe (not shown, for graph clarity), and they showed exactly the same pattern with a slight shift of 0.3-0.4°C all along the curve. Note that Pool2 is globally warmer than Pool1 over two years; that Pool1 seems to undergo more fluctuations than Pool2; and that different parameters vary in a correlated manner in Pool1 at a daily timescale.

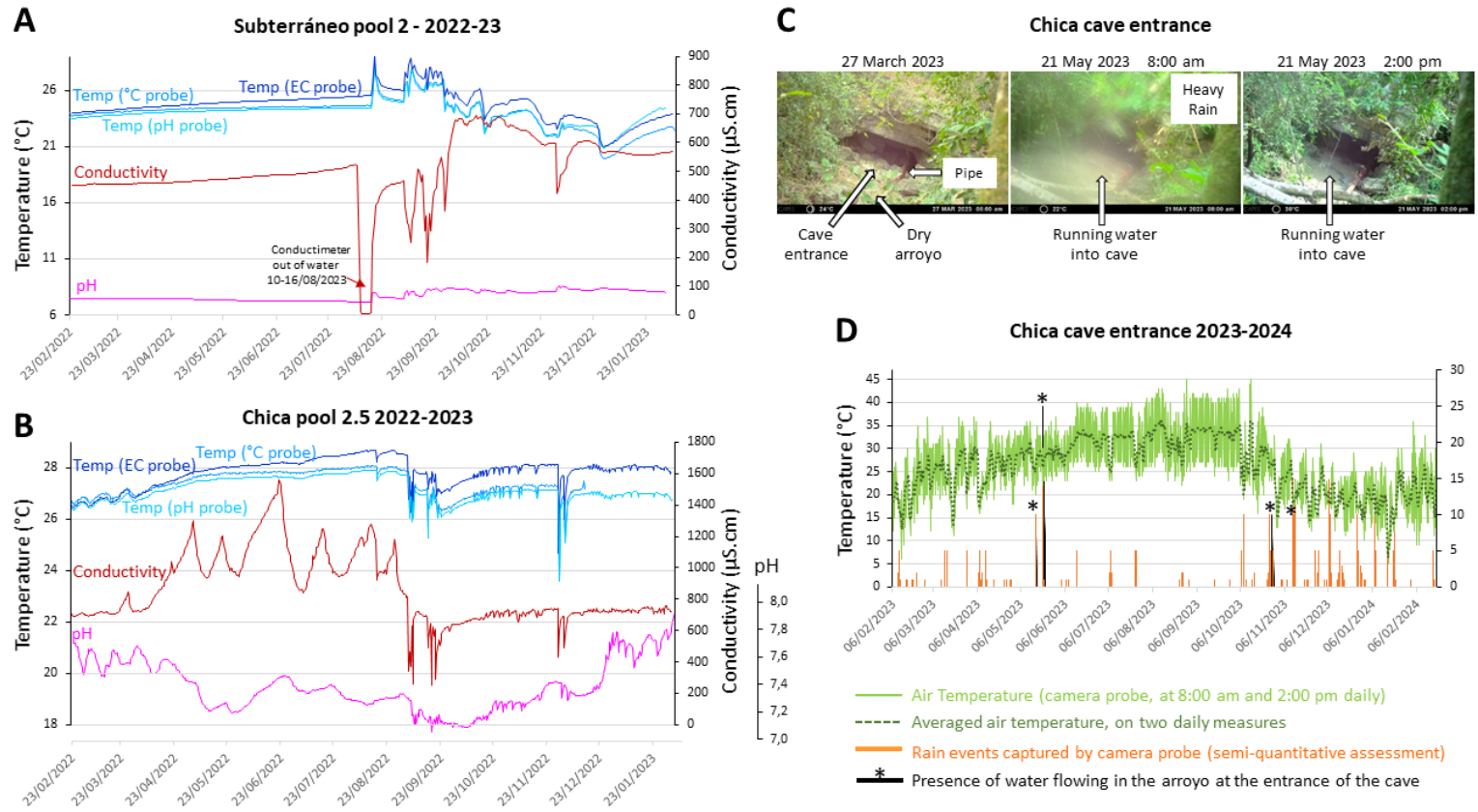


Fig7

Figure 7: longitudinal measurements of water parameters during one year in Subterráneo and Chica

A: Evolution of temperature, pH and conductivity from February 2022 to February 2023 in the Subterráneo cave pool 2. Color codes for recorded parameter are indicated. Note that water temperature recorded from the specific temperature probe, or the conductivity probe (EC), or the pH probe show identical results and temperature variation patterns. We interpret the sudden, unexplained and unrelated drop in conductivity between the 10th and the 16th October 2023 as the device being out of water. After this event, conductivity records resumed and are fully consistent as they are correlated to variations in temperature and pH at a daily timescale.

B: Evolution of temperature, pH and conductivity from February 2022 to February 2023 in Chica pool 2.5. Color codes for recorded parameter are indicated.

C: Examples of pictures captured by the camera trap facing the entrance of the Chica cave between February 2023 and February 2024. The dates are indicated.

D: Evolution of air temperature and water flow regime and rain at the entrance of the Chica cave, as captured by the camera device installed on a tree facing the cave entrance, between February 2023 and February 2024.

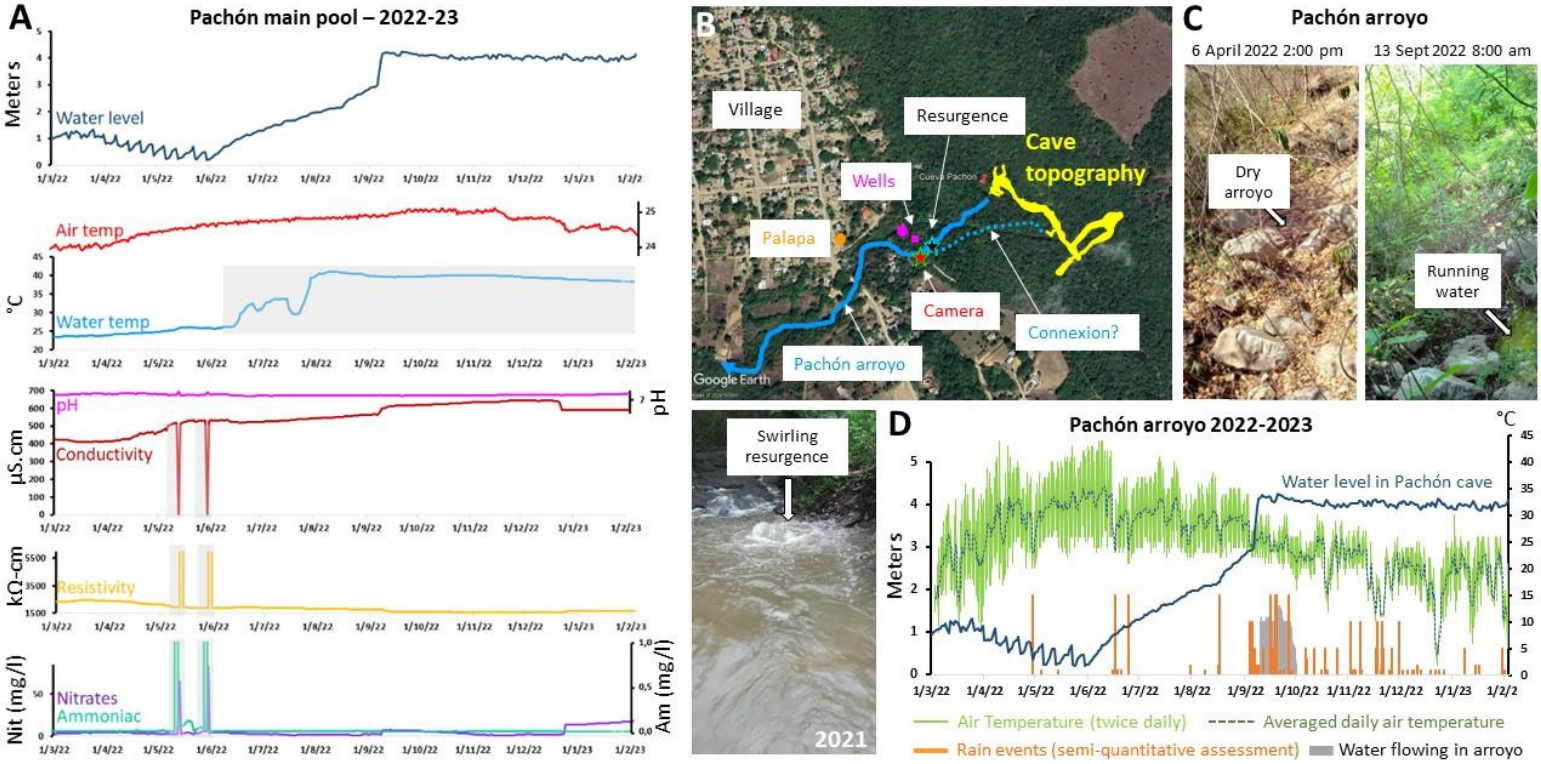


Fig8

Figure 8: longitudinal measurements of water parameters during one year in Pachón

A: Evolution of physico-chemical water and air parameters from February 2022 to February 2023 inside the Pachón cave, main pool. The grey-shaded parts of the curves should not be considered, because they result from the probes being out of the water, when levels reached extremely low values (see text).

B: Schema of the geography around the Pachón cave, superimposed on Google Earth view of the area. Relevant information is indicated. The bottom photograph taken by a local in 2021 shows the active resurgence, with important water flow.

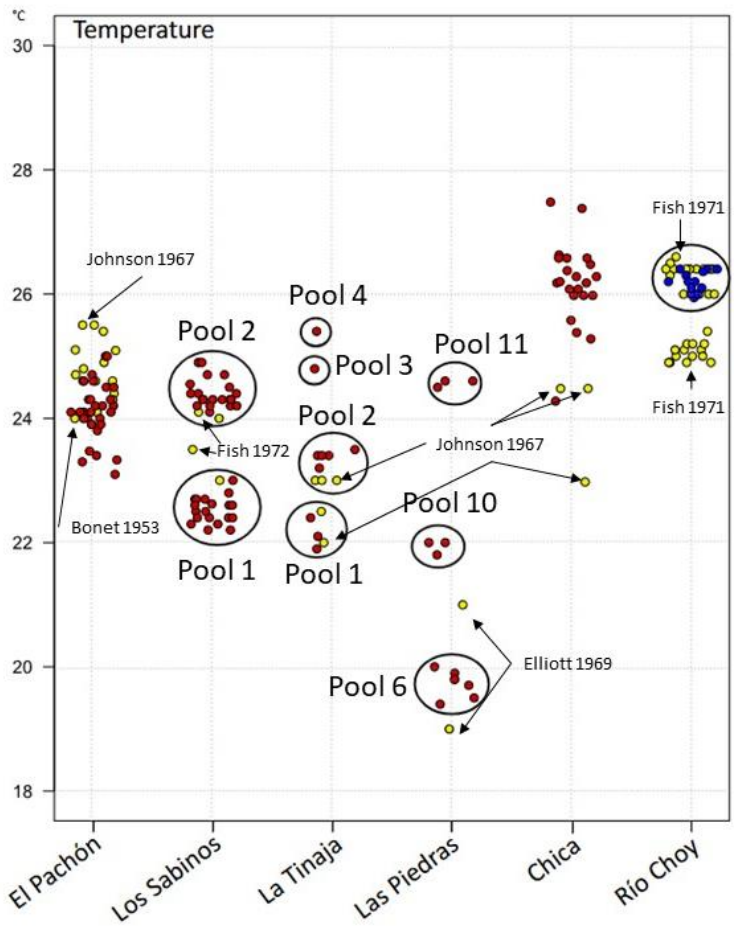
C: Examples of pictures captured by the camera trap installed along the arroyo flowing below the Pachón cave entrance. The dates are indicated. The position of the camera is shown in panel B.

D: Evolution of air temperature, rain and water flow regime at the level of the arroyo below the Pachón cave entrance, as recorded by the camera device installed on a tree along the arroyo. The water level recorded by multi-parametric probe inside the cave (panel A) is superimposed in order to correlate the different events.

Supplemental Figures and Legends

Supplemental Figure 1: Equipment used.

Photographs illustrating the state of conservation of probes and their fixation and protection systems, after one year spent in the natural cave environment. Photos by LL.



Suppl Fig2

Supplemental Figure 2: Stability of water temperature measurements over 7 decades (1953-2024)

Same as Figure 3, including also data collected for Cueva de Los Sabinos, Cueva de la Tinaja, Sótano de Las Piedras, Cueva Chica. Red dots (caves) and blue dots (river) are from our dataset. Black circles indicate cave pool numbers, known from our dataset. Yellow dots are from ancient literature, with the author name & date. However, in most cases there is no pool location information in these reports. Older measurements are in the range of ours, suggesting global stability of mean temperatures in these caves over 70 years.

For the surface station Nacimiento del Río Choy (blue dots; river location #3 on **Fig.1A**), a black circle shows a group of ours & old data (Fish, 2004) close to 26°C. Another group of only older data is close to 25°C, also from Fish (2004). Fish showed that temperature could vary within days with flooding (e.g., between 5 June and 14 August 1971) at this location, with also massive variations in discharge, turbidity and water chemistry.

Suppl Fig3

Supplemental Figure 3: Recordings of water temperature in Pachón in 2019

Evolution of temperature from 15 March 2019 to 25 December 2019 in the main and lateral pools of the Pachón cave (Hobo probes). Recordings lasted 4 months in the main pool and 9 months in the lateral pool. Contrarily to other caves, the temperature appears very stable, varying mostly between 24°C and 25°C.